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decreased by 30% (P < 0.01) after the fish oil/evening primrose oil mixture. Atherogenic index decreased by 12% (P < 0.05) after fish oil/evening primrose oil and by 6% (P = ns) after fish oil alone. This difference was statistically significant (P < 0.05). Plasma homocysteine was reduced by 10% (P < 0.05) after the fish oil/evening primrose oil mixture and decreased 4% (P = ns) after the fish oil alone. Plasma fibrinogen decreased after both oils. The combined oils did not raise plasminogen activator inhibitor-1 (PAI-1) **antigen** at all, whereas after fish oil there was a 49% (P < 0.05) increase. Fish oil increased the ratio C20:4 to C20:3, an index of delta-5-desaturase, by 96% (P < 0.001) and reduced the ratio of C20:3 to C18:2, an index of delta-6-desaturase, by 38% (P < 0.001), whereas the fish oil/evening primrose oil mixture left these indexes unchanged. A high index of delta-5-desaturase has been found to be correlated to increased **insulin** sensitivity. In conclusion, combination of fish oil and evening primrose oil had a more favorable effect on the atherogenic index and caused no increase in PAI-1 **antigen**. The effects on **triglycerides** and PAI-1 of the fish oil and the mixture appears to be a result of their (n-3) **fatty acid** content.

L85 ANSWER 19 OF 53 MEDLINE
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Time Course of Increased Plasma Cytokines, Cortisol, and Urea Nitrogen in Pigs Following Intraperitoneal Injection of Lipopolysaccharide[1]

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ABSTRACT: The emerging view is that reduced feed intake, lean muscle accretion, and growth in immunologically challenged pigs is the result of increased cytokine activity, but this has not been directly tested. To begin addressing this issue, 72 crossbred barrows and gilts (11.55 ± .19 kg BW) were not fed for 12 h and then injected i.p. with 0, .5, or 5 μg/kg of *Escherichia coli* lipopolysaccharide (LPS). Blood was collected by jugular puncture at 0, 2, 4, 8, 12, and 24 h after injection. Plasma levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), cortisol, plasma urea nitrogen (PUN), NEFA, and triglycerides were determined. Immunological stress was induced by LPS as indicated by increased secretion of TNF-α, IL-6, and cortisol. In pigs receiving 5 μg/kg of LPS, plasma TNF-α was increased 10-fold at 2 h after injection and was still elevated ($P < .01$) at 4 h. In these same pigs, plasma concentration of IL-6 was increased at 2 h and peaked at 4 h with levels exceeding baseline values by 200-fold ($P < .01$). Cortisol was elevated at 2, 4, and 8 h after injection ($P < .01$). The increased secretion of cytokines and cortisol in pigs injected with 5 μg/kg of LPS was followed by an increase in protein degradation, as evidenced by PUN values that were increased two- and threefold at 8 and 12 h after injection, respectively. However, unlike previous reports in laboratory animal species, plasma glucose, NEFA, and triglycerides were not altered by LPS. Nonetheless, as the period of feed deprivation progressed from 12 to 36 h, plasma NEFA and triglycerides increased ($P < .05$) and plasma glucose tended to decrease. We believe that immunological challenge induces cytokine synthesis and secretion in swine which, in turn, may induce protein catabolism.

Key Words: Cytokines, Cortisol, Pigs, Lipopolysaccharides

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Introduction

Considerable evidence indicates that the metabolic effects characterizing immunological challenge are important for maintaining homeostasis during infection (Klasing and Johnstone, 1991; Adi et al., 1992). Those responses are attributed to cytokines that are released by activated macrophages. The proinflammatory cytokines that have profound metabolic effects in rodents include tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) (Memon et al., 1994). Because porcine macrophages produce the same array of cytokines (Murtaugh, 1994), the emerging view is that the reduction in feed intake and growth observed in diseased or immunologically challenged pigs is the result of increased cytokine activity (Kelley et al., 1994).

It is apparent from studies comparing the growth of pigs reared in highly sanitized facilities to those reared in more conventional environments that immunological stress can reduce growth rate and feed efficiency from 10 to 25% (Williams et al., 1993; Coffey and Cromwell, 1995). Although this economically important problem has been tentatively linked to the proinflammatory cytokines, neither their synthesis or secretion nor the metabolic changes following immune challenge have been studied in swine. Therefore, the objectives of the present study were to characterize plasma levels of TNF-α, IL-6, cortisol, and several metabolites that are indicative of changes in nutrient metabolism.

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Materials and Methods

Animals and Management. Pigs from the University of Illinois Swine Research Center resulting from the cross of PIC (Franklin, KY) Line-26 males and

Camborough-15 females were used. They were housed in an environmentally controlled building with constant 24-h lighting. The pigs were maintained three pigs per pen in 1.2-m^2 raised wire floor pens equipped with nipple waterers that allowed free access to water. To allow for adaptation to the environment, pigs were assigned to pens 7 d before experimentation. All procedures and housing were approved by the University of Illinois Committee on Laboratory Animal Care.

Blood Collection and Analysis. Blood samples were collected aseptically from the cranial vena cava region into lithium heparinized syringes and centrifuged promptly (2,000 × *g* for 10 min at 5°C). Aliquots of plasma from each pig were stored at −23°C until analysis could be conducted.

Total plasma cortisol was measured using a commercially available ^{125}I RIA kit (ICN Biomedicals, Costa Mesa, CA). Recovery was validated using 20 μL of porcine plasma supplemented with 0, 10, 100, or 1,000 ng/mL of cortisol standards in a 25-μL reaction volume. Recoveries ranged from 82 to 100%. Plasma samples were serially diluted 1:2, 1:4, and 1:8 with the provided diluent to demonstrate parallelism. Intraassay variation was 2.4%, and sensitivity of the assay was 1.5 ng/mL.

Total plasma TNF-α was measured using a commercially available ELISA specific for porcine TNF-α (Endogen, Cambridge, MA). Plasma samples were assayed in duplicate at either 1:1 or 1:10 dilution. The assay was sensitive to 10 pg/mL of TNF-α and had an intraassay CV of < 10%.

Total plasma IL-6 was measured using the IL-6 sensitive, 7td1 B-cell hybridoma cell proliferation assay (Wright et al., 1993). Cells were suspended at a concentration of 1.5 × 10^5 cells/mL in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 100 U/mL of penicillin and 100 mg/mL of streptomycin. Fifty microliters of cell suspension was added to each well of a 96-well plate. Fifty microliters of RPMI-1640 containing serum (diluted 1:50, 100, 200, 400, 800, and 1,600) or recombinant mouse IL-6 (200 pg/mL and six 1:2 dilutions of this amount down to .095 pg/mL) was added to each well and assayed in triplicate. After 72 h at 37°C, 5% CO_2, and 95% humidity, proliferation was determined by adding 40 μL of 3-(4,5-dimethlthiazol-2-yl),-2,5-diphenyltetrazolium bromide (**MTT**; 2 mg/mL in double deionized water). The MTT was incubated with the cells at 37°C for 4 h, at which time 100 μL of 50% (vol/vol) dimethyformamide (**DMF**):20% (wt/vol) SDS dissolved in double deionized water was added to each well. Cells were incubated for 16 h with the 50% DMF:20% SDS solution to lyse cells and solubilize MTT crystals. Plates were read at 550 nm using an automated microplate reader (EL311, Bio Tek Instruments, Winooski, VT). The absorbance of triplicate wells was averaged and compared to standard curve values to determine units of IL-6 activity.

Plasma samples were analyzed for α-1 acid-glycoprotein (**α-1-AGP**) concentrations using a radial immunodiffusion assay (Development Technologies International, Frederick, MD). Aliquots of 5 μL of plasma were pipetted into agar plates containing antibodies specific for porcine α-1-AGP and incubated for 48 h at 37°C. The concentration of α-1-AGP was determined by measuring the precipitin ring formed in the agar and comparing it to a standard curve developed using purified porcine α-1-AGP.

Plasma triglycerides were measured using the GPO-Trinder method, an enzymatic, colorimetric assay (Sigma Chemical, St. Louis, MO). Plasma NEFA were measured using an enzymatic, colorimetric assay purchased from Wako Chemical (Norfolk, VA). Plasma glucose concentration was measured using an enzymatic, colorimetric assay (Sigma). Plasma urea nitrogen was measured on an autoanalyzer (Boehring Mannheim Diagnosis, Indianapolis, IN) using a method based on the procedure of Skeggs (1957).

Experimental Design. Seventy-two pigs (36 barrows, 36 gilts) with an average initial weight of 11.6 kg were allotted to pens based on sex, ancestry, and weight. Pens consisted of three pigs of the same sex originating from the same litter, when possible. Individual pigs within pens were randomly assigned to one of three injection treatments, and each pen of pigs was randomly assigned to one of six sampling times. The design was chosen so that pigs were only sampled once, eliminating the effects of prior sampling. Thus, at each sampling time two barrow and two gilt pens were sampled (12 pigs, four pigs/treatment). Treatments (LPS injections) were administered immediately following initial weight determination at 0800. Treatments included injection of three doses of LPS: 0, .5, and 5 μg/kg BW. The LPS (*Escherichia coli* serotype K-235; Sigma) was dissolved in sterile .9% (wt/vol) NaCl solution so that injection of 1 mL/kg of solution would achieve the desired dosage. All injections were given i.p. in the lower abdominal region.

Blood samples were taken from two barrow and two gilt blocks before initial weight determination and at 2, 4, 8, 12, and 24 h after LPS injection. Blood samples taken before initial weight determination are described as time 0. All pigs were deprived of feed for 12 h before time 0, and they were not given feed during the course of the 24-h postinjection evaluation period.

Statistical Analysis. Data were analyzed as a completely randomized design, and ANOVA was conducted using the GLM procedure of SAS (1992). A factorial arrangement of treatments was used with the following factors: sex (barrow vs gilt), dose (0, .5, or 5μg/kg LPS), and time (0, 2, 4, 8, 12, or 24 h after injection). The main effects and their appropriate interactions were tested, and it was observed that the effect of sex was not significant ($P > .10$). Sex, therefore, was not included in subsequent analyses.

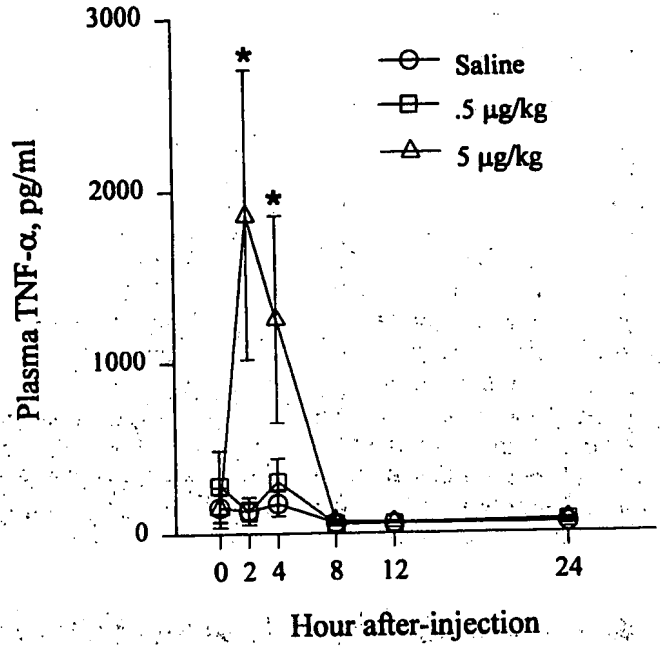

Figure 1. Plasma tumor necrosis factor-α (TNF-α) of pigs following a challenge dose of lipopolysaccharide. Pigs were injected i.p. with either saline or .5 or 5μg/kg BW of LPS a 0 h. Feed was removed 12 h before injection and was not available throughout the 24-h period after injection. Asterisks indicate that a treatment mean at a given time period is different from the saline-injected control ($P < .05$).

Data were then subjected to one- (dose, time) and two-way (dose × time) ANOVA to determine the significance of the main factors and their interactions. When ANOVA revealed a significant effect of dose or a dose × time interaction, differences among treatment means were tested using paired *t*-tests.

Results

Intraperitoneal injection of LPS increased plasma levels of TNF-α and IL-6. Two-way ANOVA of plasma TNF-α and IL-6 concentrations revealed an effect of dose ($P < .01$), time ($P < .01$), and a time × dose interaction ($P < .01$). Whereas .5 μg/kg LPS did not increase plasma TNF-α, 5 μg/kg LPS induced a 10-fold ($P < .01$) increase in plasma TNF-α at 2 h after the injection (Figure 1). In these pigs, plasma TNF-α was still elevated at 4 h ($P < .01$) but returned to baseline by 8 h after injection (Figure 1). Concomitant with the peak in TNF-α, the plasma concentration of IL-6 in pigs receiving 5 μg/kg LPS was increased ($P < .01$), although .5 μg/kg LPS had no effect on IL-6 levels (Figure 2). Plasma IL-6 levels of pigs receiving 5 μg/kg LPS peaked at 4 h after injection at levels greater than 200-fold ($P < .01$) those of the controls (Figure 2). Plasma IL-6 levels of LPS-treated pigs returned to normal by 12 h after injection.

Administration of LPS increased the activity of the hypothalamic-pituitary-adrenal axis, as evidenced by increased plasma cortisol levels (Figure 3). Two-way ANOVA of plasma cortisol concentration revealed an effect of time ($P < .01$) and dose ($P < .01$) and a time × dose interaction ($P < .01$). Plasma cortisol before injection was between 2.5 and 3.5 μg/dL, which is consistent with previous observations of nonstressed pigs (Johnson et al., 1994). Both doses of LPS increased ($P < .05$) plasma cortisol concentrations at 2 h after injection, but the change in plasma cortisol induced by .5 μg/kg was smaller and more transient than that induced by 5 μg/kg LPS. Plasma cortisol concentration of pigs injected with 5 μg/kg LPS peaked at 4 h and remained elevated ($P < .05$) at 8 h before returning to control levels at 12 h after injection.

Plasma urea nitrogen, an indicator of protein catabolism in feed-deprived animals, was elevated by LPS administration (Figure 4). The PUN levels were increased two- and threefold ($P < .05$) at 8 and 12 h, respectively, in pigs injected with 5 μg/kg LPS. Administration of .5 μg/kg LPS had no effect on PUN levels. There was a trend ($P < .10$) for increased PUN levels at 24 h after injection that could be explained by proteolysis typical of an extended period without feed.

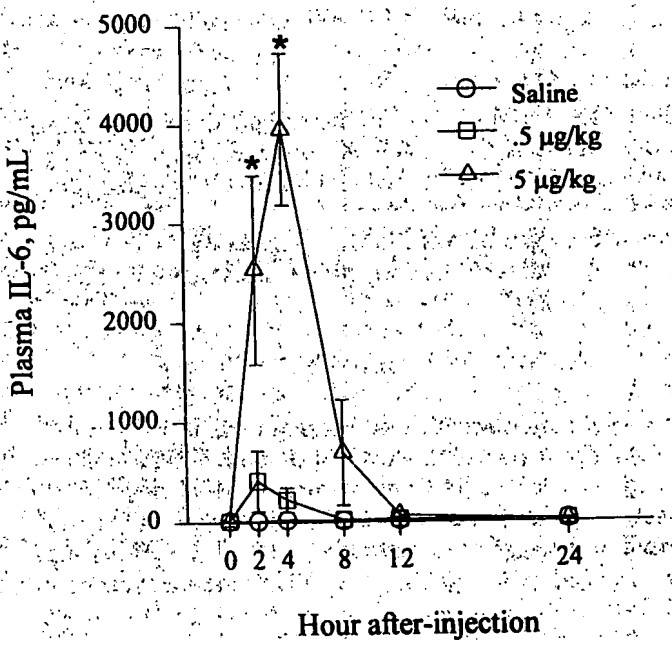

Figure 2. Plasma interleukin-6 (IL-6) of pigs following a challenge dose of lipopolysaccharide. Pigs were injected i.p. with either saline or .5 or 5 μg/kg BW of LPS at 0 h. Feed was removed 12 h before injection and was not available throughout the 24-h period after injection. Asterisks indicate that a treatment mean at a given time period is different from the saline-injected control ($P < .05$).

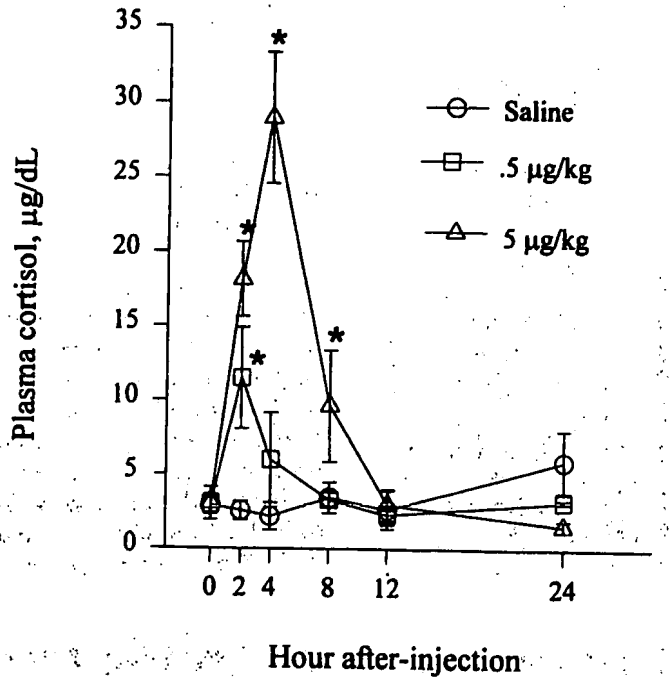

Figure 3. Plasma cortisol of pigs following a challenge dose of lipopolysaccharide. Pigs were injected i.p. with either saline or .5 or 5 μg/kg BW of LPS at 0 h. Feed was removed 12 h before injection and was not available throughout the 24-h period after injection. Asterisks indicate that a treatment mean at a given time period is different from the saline-injected control ($P < .05$).

Pigs lost an average of 800 g of BW due to the extended period of feed deprivation, but LPS injection had no effect on weight loss ($P > .10$). Changes in plasma concentrations of glucose, triglycerides, NEFA, and α-1-AGP were not significant (Table 1). However, the duration of feed deprivation seemed to affect those variables. As expected, NEFA and triglyceride concentrations increased ($P < .05$) and plasma glucose tended to decrease with time of feed deprivation.

Discussion

The present view is that the reduction in feed intake, lean muscle accretion, and growth observed in diseased or immunologically challenged pigs is the result of increased cytokine activity, but this has not been directly tested. As a first step toward addressing this issue, pigs were injected i.p. with three doses of LPS in order to activate the immune system and develop complete dose- and time-response curves for the cytokines TNF-α and IL-6, cortisol, and several blood metabolites that may indicate changes in protein, lipid, and carbohydrate metabolism. The resultant data demonstrate that pigs injected with LPS have increased plasma levels of TNF-α, IL-6, and cortisol. Furthermore, the increase in circulating cytokines and cortisol caused by LPS was followed by a dramatic increase in PUN levels, but not triglycerides or NEFA. Because PUN was elevated by LPS in feed-deprived animals, the present results are interpreted to suggest that immunological challenge increases muscle protein degradation, perhaps via the induction of macrophage-derived cytokines.

There is substantial evidence to suggest that cytokines synthesized in response to challenge by LPS either directly or indirectly cause skeletal muscle protein degradation. In rodents, LPS increases plasma levels of TNF-α, IL-6, and IL-1, and it also increases skeletal muscle protein degradation (Jepson et al., 1986; Fong et al., 1989; Goodman, 1991). This is also the case in birds; increased IL-1-like activity in plasma has been reported in chicks injected with LPS (Klasing et al., 1987). Additionally, when incubated with a crude preparation of IL-1 in vitro, increased proteolysis was observed in skeletal muscle isolated from the chick wing (Klasing et al., 1987). Numerous studies involving injections of recombinant cytokines including TNF-α, IL-1, and IL-6 now confirm that the effects of LPS on protein metabolism are due in large part to the induction of cytokine synthesis.

Intravenous infusion of TNF-α alone or in combination with IL-1 increased skeletal muscle protein

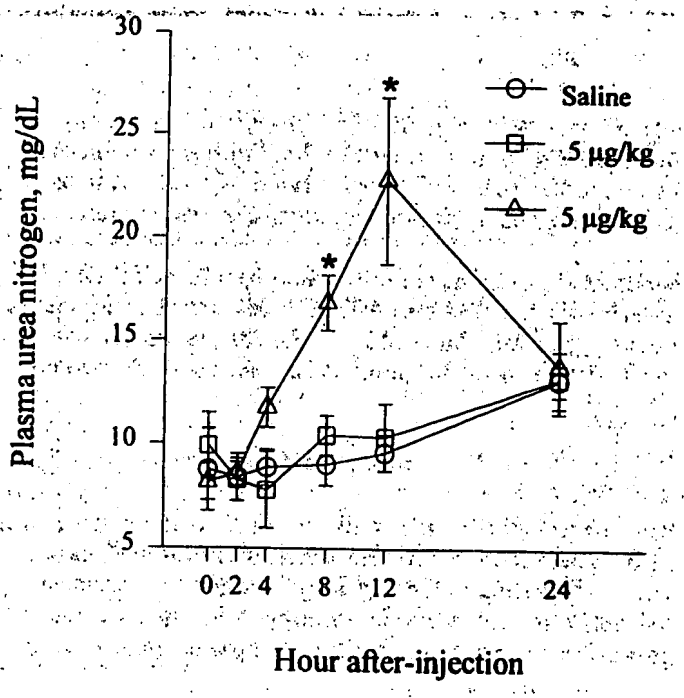

Figure 4. Plasma urea nitrogen of pigs following a challenge dose of lipopolysaccharide. Pigs were injected i.p. with either saline or .5 or 5 μg/kg BW of LPS at 0 h. Feed was removed 12 h before injection and was not available throughout the 24-h period after injection. Asterisks indicate that a treatment mean at a given time period is different from the saline-injected control ($P <$ .05).

Table 1. Plasma concentration of various blood metabolites and α-1-acid glycoprotein following a challenge dose of lipopolysaccharide[a]

Variable and LPS[b], μg/kg	Hour after injection						SEM
	0	2	4	8	12	24	
NEFA, meq/dL							
0	160.9	528.3	406.7	801.0	812.3	730.5	50.3
.5	252.9	728.3	341.7	840.3	902.2	652.7	48.9
5	270.5	543.4	526.0	644.6	680.8	609.9	51.6
Triglyceride, mg/dL							
0	54.8	53.3	68.2	66.7	73.4	86.2	5.2
.5	62.5	42.6	56.1	50.5	92.4	106.6	5.0
5	45.6	58.6	88.8	52.2	64.2	103.8	5.3
Glucose, mg/dL							
0	82.2	67.2	58.9	69.1	64.7	71.6	3.6
.5	80.3	67.8	77.8	59.1	51.0	64.7	3.5
5	77.2	74.7	47.2	86.6	53.5	71.4	3.7
α-1-AGP, μg/mL[c]							
0	1,117	1,123	1,266	1,316	1,070	1,058	35.7
.5	1,025	1,050	1,065	1,281	1,076	1,100	34.7
5	1,077	1,118	1,065	1,073	1,076	976	36.6

[a]Data represent least squares means of four pigs; average weight was 11.55 kg.
[b]LPS = lipopolysaccharide (*Escherichia coli*, serotype K-235). Three doses of LPS (0, .5, or 5 μg/kg BW) were injected i.p. at time 0 and blood samples were taken at 0, 2, 4, 8, 12, and 24 h following injection.
[c]α-1-AGP = α-1-acidglycoprotein.

catabolism in rats (Flores et al., 1989). In addition, Goodman (1991) showed that infusion of TNF-α and LPS, but not IL-1, enhanced muscle proteolysis, as evidenced by 3-methyl-L-histidine and tyrosine release from isolated skeletal muscle and an increase in blood urea concentrations. Consistent with the idea that cytokines secreted by activated macrophages induce muscle protein degradation, the increase in PUN at 8 h in pigs given 5 μg/kg of LPS occurred only after peak levels of TNF-α (i.e., at 2 h) and IL-6 (i.e., at 4 h) were observed. The time course of increased plasma cytokines, cortisol, and urea nitrogen after i.p. injection of 5 μg/kg LPS is shown in Figure 5. However, pigs that received .5 μg/kg of LPS did not have increased plasma levels of TNF-α or IL-6, and they did not have increased levels of PUN. Interestingly, we have recently reported that this same dose of LPS induced profound anorexia even in pigs deprived of feed for 12 h (Johnson and von Borell, 1994; Warren et al., 1997). Therefore, it seems reasonable to postulate that increased muscle catabolism, indicated in this study by a profound threefold increase in PUN, was due to the elevated levels of plasma cytokines.

Although the proinflammatory cytokines act directly on skeletal muscle to inhibit protein accretion and accelerate protein degradation, cytokines also induce a variety of other endocrine responses that are likely to induce proteolysis. For example, TNF-α, IL-6, and IL-1 stimulate the hypothalamic-pituitary-adrenal axis. In particular, IL-1 has been shown to stimulate neurons in the hypothalamus to secrete corticotropin-releasing hormone (Berkenbosch et al., 1987). Moreover, infusion of IL-1 receptor antagonist into a lateral ventricle of the rat brain inhibited the expression of corticotropin-releasing hormone mRNA in the hypothalamus following i.p. injection of LPS (Kakucska et al., 1993). The secretion of glucocorticoids is part of a negative feedback loop that regulates the immune system to prevent it from overreacting (Knudson et al., 1987; Bertini et al., 1988; McCallum et al., 1990; Johnson et al., 1996). However, glucocorticoids also produce a variety of tissue-specific metabolic effects. In liver they are anabolic in that they increase gluconeogenesis and protein synthesis, but in muscle and adipose tissue, glucocorticoids are catabolic, inducing proteolysis and lipolysis, respectively (Millward et al., 1985; Lacasa et al., 1988). Because LPS increased plasma cortisol in a time- and dose-dependent fashion in our study, it is plausible that they contributed to the increased PUN following LPS administration.

The amino acids liberated from muscle protein degradation during inflammation are thought to provide fuel for hepatic acute phase protein synthesis (Reeds et al., 1994). The cytokine IL-6 acts directly on hepatocytes to stimulate amino acid uptake and synthesis of a broad array of acute-phase proteins. The synthesis of acute-phase proteins may increase 25% or more following tissue damage or infection. Numerous acute-phase proteins have been identified in pigs, including C-reactive protein, haptoglobin, and α-1-AGP (Itoh et al., 1992; Lampreave et al., 1994). Increased serum haptoglobin concentrations have been reported in pigs infected with *Actinobacillus pleuropneumonia* (Hall et al., 1992) and elevated plasma C-reactive protein concentrations have been demonstrated in pigs injected with turpentine (Burger et al., 1992). More recently, Williams et al. (1993)

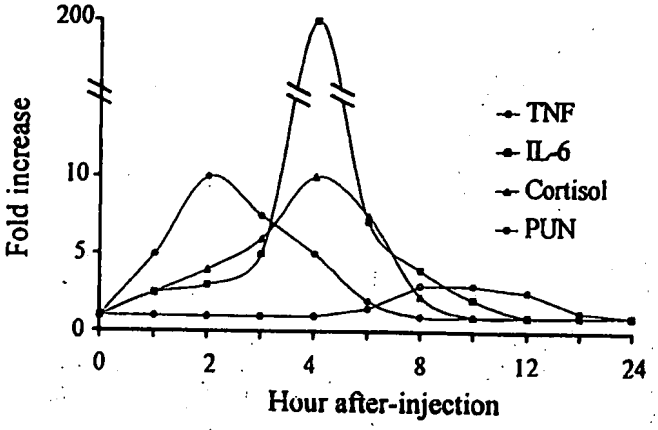

Figure 5. The time course of elevated plasma levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), cortisol, and plasma urea nitrogen (PUN) following i.p. injection of lipopolysaccharide (5 μg/kg BW). Note the threefold increase in plasma urea nitrogen after the marked increases in TNF-α and IL-6.

compared α-1-AGP levels of pigs in environments that presumably presented either a high or low level of immune stimulation and reported the level of immune stimulation and plasma concentration of α-1-AGP to be positively correlated. In the present study, however, despite increasing plasma TNF-α, IL-6, and cortisol, LPS did not increase α-1-AGP concentrations. It should be noted that the baseline levels of α-1-AGP for pigs in our study were similar to those reported by Williams et al. (1993) for pigs that presumably had a high level of immune stimulation. Indeed, in their study plasma level of α-1-AGP was used as an indicator of immune system activation. Thus, if pigs in the present study had maximal levels of α-1-AGP before LPS, this may explain why α-1-AGP was not increased by LPS. Alternatively, other investigators reported that α-1-AGP levels were not elevated in the plasma of pigs following the injection of turpentine (Lampreave et al., 1994) or LPS (M. E. Spurlock, personal communication), suggesting that acute immunological stress, induced by turpentine or LPS, does not increase the synthesis of α-1-AGP.

Alterations in lipid and glucose metabolism are also associated with infection. In rodents, TNF-α, IL-6, and IL-1 induce hypertriglyceridemia by decreasing muscle and adipose lipoprotein lipase activity and by increasing the rate of hepatic fatty acid synthesis and their subsequent incorporation into very-low-density lipoproteins (Hardardottir et al., 1994). However, in our study neither triglycerides nor NEFA were elevated by LPS, even though IL-6, which was increased 200-fold, has recently been shown to increase triglycerides in rats (Nonogaki et al., 1995). Species differences in the site of fatty acid synthesis could explain the apparent absence of elevated triglyceride levels in the current study because rats and pigs synthesize fat *de novo* primarily in liver and adipose tissue, respectively.

Alterations in glucose metabolism are associated with immunological challenges, but the observed changes can be highly variable depending on the severity of insult and the time of sampling (Lang and Spitzer, 1987). In general, immunological stress increases hepatic glucose production via increases in gluconeogenesis and glycogenolysis while at the same time increasing extrahepatic utilization of glucose. The net result of these changes is an early, transient hyperglycemia. Lang et al. (1992) reported elevated plasma glucose concentrations following LPS injection that returned to normal values by 2 h after the injection. It is possible that we did not see altered plasma glucose in our study because the first sampling time was too late to observe this change.

Regarding the effects of feed deprivation per se, plasma concentrations of NEFA and triglycerides increased from 0 to 24 h after injection (12 to 36 h of feed deprivation). Elevated NEFA levels are common in feed-deprived animals as a result of increased adipose tissue lipolysis (Murray et al., 1993). Plasma triglycerides also increase during feed deprivation because plasma NEFA concentrations exceed the capacity of liver to oxidize them. An increase in protein breakdown was also evident at 24 h after injection as indicated by an elevation in PUN. Increased protein degradation is a typical response in early feed-deprived conditions because amino acids are needed as gluconeogenic substrates in the liver.

In conclusion, LPS administration triggered the release of TNF-α, IL-6, and cortisol. Because these molecules have been shown to induce proteolysis and lipolysis in other species, it seems reasonable to postulate that they play an important role in modulating growth in immunologically challenged pigs. More specifically, cytokines may have a direct role in inducing elevated muscle protein catabolism, as evidenced by the elevated PUN concentrations observed herein.

Implications

The present study indicates that stimulation of the pig's immune system results in increased plasma levels of cytokines and cortisol. These metabolically active molecules may be involved in the reduction of feed intake, lean muscle accretion, and growth in diseased or immunologically challenged pigs. Because the increase in cytokines and cortisol was followed by a profound increase in plasma urea nitrogen, these data support the idea that immunological challenge induces skeletal muscle protein degradation. Therefore, we suggest that understanding how cytokines alter intermediary metabolism in growing animals is a prerequisite to understanding why sick or immune-challenged animals do not grow well.

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insulin sensitivity in liver, glycaemic control, lipoprotein profile and fibrinolysis in postmenopausal women with NIDDM. For a definite answer as to whether oestrogens can be more liberally used in NIDDM patients, long term studies including the effect of progestogens are necessary.

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97173401 EMBASE Document No.: 1997173401. Dietary fish oil enhances circulating **interferon**-.gamma. in mice during listeriosis without altering in vitro production of this cytokine. Fritsche K.L.; Feng C.; Berg J.N.. Dr. K.L. Fritsche, 110 Animal Sciences Research Center, University of Missouri, Columbia, MO 65211, United States. Journal of Interferon and Cytokine Research 17/5 (271-277) 1997.
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AB The objective of this study was to investigate the impact of feeding mice a diet rich in n-3 polyunsaturated **fatty acids** (PUFA) from fish oil on the **interferon**-.gamma. (IFN-.gamma.) response during an active infection with Listeria monocytogenes. Weanling female C3H/Hen mice were fed experimental diets containing 20% by weight one of the following fats: **soybean oil**, lard, or a mixture of menhaden fish oil and **corn oil** (17:3, w/w). After 4 weeks, mice were **injected** with 105 live L. monocytogenes, and the concentration of IFN- .gamma. in serum and spleen was determined 0, 2, 4, and 7 days postinfection by enzyme-linked immunosorbent assay (ELISA). Fish oilfed mice showed significantly higher IFN-.gamma. in their blood at 2 and 4 days postchallenge compared with mice fed the **soybean oil**-containing or lard-containing diets ($p < 0.001$). A higher concentration of IFN-.gamma. was also found in the spleen homogenate of fish oil-fed mice on day 4 postchallenge ($p < 0.005$). To examine in vitro IFN-.gamma. production, splenocytes were isolated from fish oil- fed and **soybean oil**-fed mice on day 4 postchallenge and cultured with concanavalin A (1 .mu.g/ml and 10 .mu.g/ml) for 24 and 48 h. There were no significant differences in the IFN-.gamma. concentration in cell culture supernatants between these diet treatments. This study demonstrated that the elevation in the concentration of IFN-.gamma. in blood and spleen during murine listeriosis is accentuated and prolonged by dietary n-3 PUFA, and these effects may not be due to changes in IFN-.gamma. production.

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Dietary Fish Oil Enhances Circulating Interferon-γ in Mice During Listeriosis Without Altering *In Vitro* Production of This Cytokine

K.L. FRITSCHE,[1] C. FENG,[1] and J.N. BERG[2]

ABSTRACT

The objective of this study was to investigate the impact of feeding mice a diet rich in n-3 polyunsaturated fatty acids (PUFA) from fish oil on the interferon-γ (IFN-γ) response during an active infection with *Listeria monocytogenes*. Weanling female C3H/Hen mice were fed experimental diets containing 20% by weight one of the following fats: soybean oil, lard, or a mixture of menhaden fish oil and corn oil (17:3, w/w). After 4 weeks, mice were injected with 10^5 live *L. monocytogenes*, and the concentration of IFN-γ in serum and spleen was determined 0, 2, 4, and 7 days postinfection by enzyme-linked immunosorbent assay (ELISA). Fish oil-fed mice showed significantly higher IFN-γ in their blood at 2 and 4 days postchallenge compared with mice fed the soybean oil-containing or lard-containing diets ($p < 0.001$). A higher concentration of IFN-γ was also found in the spleen homogenate of fish oil-fed mice on day 4 postchallenge ($p < 0.005$). To examine *in vitro* IFN-γ production, splenocytes were isolated from fish oil-fed and soybean oil-fed mice on day 4 postchallenge and cultured with concanavalin A (1 μg/ml and 10 μg/ml) for 24 and 48 h. There were no significant differences in the IFN-γ concentration in cell culture supernatants between these diet treatments. This study demonstrated that the elevation in the concentration of IFN-γ in blood and spleen during murine listeriosis is accentuated and prolonged by dietary n-3 PUFA, and these effects may not be due to changes in IFN-γ production.

INTRODUCTION

CLINICAL DATA HAVE SHOWN that fish oils rich in long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) have beneficial effects on inflammatory and autoimmune disorders, such as rheumatoid arthritis,[1] asthma,[2] psoriasis,[3] ischemia-reperfusion injury,[4] and cystic fibrosis.[5] During the last decade, numerous studies have demonstrated that n-3 PUFA can modulate a wide range of immune responses, including lymphocyte proliferation,[6,7] cytotoxicity,[8,9] delayed-type hypersensitivity reactions,[10] and antigen presentation.[11,12] Although it is widely recognized that dietary fats can alter immune and inflammatory responses, our understanding of how dietary lipids change the immune response is incomplete. There are three commonly proposed mechanisms by which n-3 PUFA modulate immune/inflammatory responses: changes in eicosanoid biosynthesis, alterations in membrane phospholipid fatty acid composition, which in turn could alter membrane-associated protein and receptor function, and changes in cytokine production (see reviews in refs. 13–16). Most of the fatty acid-cytokine research to date has focused on the effects of dietary n-3 PUFA on the *in vivo* and *in vitro* production of interleukin-1β (IL-1β), tumor necrosis factor-α (TFN-α), IL-6, and IL-2.[17–19] However, little is known about the effect of dietary fat, especially n-3 PUFA, on interferon-γ (IFN-γ) production.

IFN-γ is a multifunctional protein that plays a central role in modulating immune and inflammatory responses.[20,21] IFN-γ is produced by T lymphocytes and natural killer (NK) cells. It can influence the class of antibody produced by B cells, upregulate the expression of major histocompatibility complex

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Some of these data were presented at the Experimental Biology meeting held in Washington, DC, on April 14–18, 1996 [Feng, C., and Fritsche, K. (1996). Influence of dietary fat on interferon-gamma F(IN-γ) production during murine listeriosis. FASEB J. **10**, A555].

(MHC) class I and II antigens, and increase macrophage-mediated killing of intracellular pathogens. In a previous study, our laboratory reported that dietary fat influenced the expression of MHC class II molecules during murine *Listeria monocytogenes* infection.[12] Mice fed the fish oil-containing diet (rich in n-3 PUFA) showed reduced expression of MHC class II molecules compared with those fed diets rich in n-6 PUFA, monounsaturated fatty acids, or saturated fatty acids. Others have reported reduced expression of MHC class II molecules on immune cells from rats[22] and humans[23] following n-3 PUFA enrichment. MHC class II molecules are critically important for the development and function of cells in the immune system.[24–26] The expression of MHC class II molecules is inducible and influenced by many factors. Among these factors, IFN-γ is the most potent stimulator of the expression of MHC class II molecules[27,28] and prostaglandin E_2 (PGE_2) an important downregulator.[29,30] That fish oil feeding leads to reduced MHC class II expression in the face of reduced PGE_2 production is paradoxical. One possible explanation might be that fish oil reduces IFN-γ production, which might have a greater net effect on MHC class II expression than the reduced PGE_2.

In the present study, we examined the effect of dietary fat type on IFN-γ concentration in the circulation and spleen during murine listeriosis, as well as the *in vitro* secretion of IFN-γ by mitogen-stimulated splenocytes. We hypothesized that feeding mice a dietary fat source rich in n-3 PUFA (i.e., menhaden fish oil) would reduce IFN-γ production *in vivo* and *in vitro*. To test our hypothesis, we carefully formulated experimental diets using purified ingredients, such that the macronutrient and micronutrient compositions of all diets were identical, except for the source of fat. This approach is superior to the addition of test fats to commercial diets, an approach that would lead to a substantial dilution of essential nutrients on a per calorie basis.[31–33] Our long-term goal is to clearly delineate the mechanism(s) for the immunomodulatory actions of dietary n-3 PUFA. This is the first study to demonstrate that dietary n-3 PUFA affect the *in vivo* IFN-γ response in mice during an infectious disease challenge.

MATERIALS AND METHODS

Animals and diets

Specific pathogen-free weanling female C3H/Hen mice (Charles Rivers Inc., Portage, MI) were used for this study. Mice were placed in hanging wire stainless steel cages (one animal per cage) in an environmentally controlled room (21–24°C, 50%–60% relative humidity). A diurnal light cycle of 12 h was maintained throughout the study. Animals had free access to distilled water. After adaptation for 1–2 days, mice were randomly allotted to one of the three experimental diets. Housing, handling, and sample collection procedures conformed to policies and recommendations of the University of Missouri's Laboratory Animal Care Advisory Committee.

The experimental diets were designed according to the AIN-93 diet guidelines,[34] with minor modifications necessary to accommodate an increase in caloric density as fat content was increased from 7% to 20% by weight. The composition of the experimental diets was (g/kg): casein, 230; corn starch, 354.6; α-cellulose (fiber), 57.4; L-cystine (Ajinomoto Co. Inc., Tokyo, Japan), 3.4; mineral mix (AIN-93G), 40.2; vitamin mix (AIN-93G), 11.5; sucrose, 100; choline bitartrate, 2.9; fat, 200.

Table 1. Fatty Acid Composition of Experimental Diets

	Dietary treatment groups[a]		
Fatty acids[b]	*LRD*	*SOY*	*MFO*
	(mol/100 mol)		
14:0	—	—	4.6
16:0	15.9	10.4	17.7
16:1n-7	1.1	—	9.9
18:0	19.0	4.0	3.6
18:1n-7&9	51.9	24.1	16.8
18:2n-6	10.8	54.8	12.7
18:3n-6	—	0.8	0.5
18:3n-3	0.4	6.4	1.6
20:5n-3	—	—	15.9
22:5n-3	—	—	2.9
22:6n-3	—	—	12.0

[a]Diets contained 20% (w/w) lard (LRD) and soybean oil (SOY) or 17% menhaden fish oil and 3% corn oil (MFO).
[b]Fatty acids are denoted by the number of carbons: the number of double bonds, followed by the position of the first double bond relative to the terminal methyl group (n).

Lard and soybean oil, as well as other diet ingredients, were purchased from U.S. Biochemical Corp. (Cleveland, OH) unless otherwise indicated. Menhaden fish oil (RBU-D grade) was donated by Zapata Protein Inc. (Reedville, VA). To provide mice sufficient linoleic acid, an essential fatty acid, a small amount of corn oil was added to the fish oil (1:6 parts, respectively). The fat-free portion of these diets was mixed in a single batch at the beginning of the study to ensure uniformity of macronutrient and micronutrient composition across all treatment groups. Experimental diets were made by mixing small aliquots of either the lard, the soybean oil, or the fish oil-corn oil mixture (20 g) into 100 g aliquots of this fat-free diet. The fatty acid composition of the diets was analyzed by gas chromatography following extraction and acid-catalyzed methylation (Table 1). Diets were stabilized against autooxidation by the addition of 1.2 μmol/liter tertiary-butyl hydroquinone (Eastman Kodak Co.) to the oils on receipt, as well as storage of diets at 4°C. Mice were fed fresh diet daily.

Study design

Four weeks after starting the experimental diets, mice were given an intraperitoneal (i.p.) inoculation of 10^5 colony-forming units (CFU) freshly thawed *L. monocytogenes* (ATCC 43249). We have demonstrated that the dose of *Listeria* used in the present study (10^5 CFU) is not lethal.[35] Mice were killed on days 2, 4, and 7 postchallenge. Control mice were injected with sterile saline either 2 or 4 days before killing.

Sample collection

Mice were anesthetized by intramuscular (i.m.) injection of ketamine-HCl (50 μmol/100 g body weight; Aveco Co., Inc., Fort Dodge, IA) and xylazine (4 μmol/100 g body weight;

Mobay Co., Animal Health Division, Shawnee, KS). Blood was collected by cardiac puncture and allowed to clot at ambient temperature for 1 h. Serum was collected by centrifugation of blood samples (2000*g*, 20 min). Serum samples were stored at −80°C until they could be analyzed for IFN-γ.

Following blood collection, spleens were removed from mice, weighed, and placed on ice in sterile polypropylene test tubes containing 10 ml of culture medium (i.e., RPMI-1640). Within 1 h of collection, homogenates were prepared with a Dounce tissue grinder. Spleen homogenates were frozen and thawed three times and then clarified by centrifugation (2000*g*, 20 min). Supernatants were transferred into clean tubes and stored at −80°C until they could be analyzed for IFN-γ.

Production of IFN-γ by cultured splenocytes

In a separate experiment, mice were fed the same designed experimental diets for 4 weeks and then injected i.p. with 10^5 live *L. monocytogenes*. On day 4 postchallenge, spleens were harvested, and splenocytes were isolated by density gradient centrifugation. Single cell suspensions of splenocytes were made by forcing the spleen sample through a tissue sieve (Sigma, St. Louis, MO) equipped with an 80-mesh stainless steel screen. Using a 10-ml syringe without a needle, cell clumps were dispersed by several gentle washings through the sieve. Red blood cells and dead cells were removed by centrifugation (30 min at 400*g*) of the cell suspension over Histopaque-1077 (Sigma Diagnostics) at 20°C. After centrifugation, mononuclear cells at the interface (predominantly lymphocytes) were collected, washed twice, and resuspended in medium. Cell samples were enumerated electronically with a Coulter Counter, Model ZBI (Coulter Electronics, Hialeah, FL).

Splenocytes were resuspended at a concentration of 10^6 cells/ml in RPMI-1640 medium (pH 7.4) with the following additives: $NaHCO_3$ (24 mmol/liter), L-glutamine (2 mmol/liter), HEPES (10 mmol/liter), penicillin (100,000 U/liter), streptomycin (0.17 mmol/liter), 100 ml/liter fetal bovine serum (FBS). Cells were cultured in 24-well flat-bottom tissue culture plate (10^6 cells per well) at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. IFN-γ production was stimulated with concanavalin A (i.e., a mitogen) at 1 μg/ml and 10 μg/ml. Cells cultured without mitogen served as unstimulated controls. Cell-free supernatants were collected after 24 and 48 h of culture and stored at −80°C until IFN-γ concentration was measured by ELISA.

IFN-γ assay

A commercially available ELISA kit (Endogen, Cambridge, MA) was used for determination of IFN-γ concentration in the samples according to the manufacturer's instructions. Briefly, plates were treated with coating antibody overnight. The following day, assay buffer (DPBS with 2% BSA, 0.01% thimerosal, pH 7.2) was added to each well to block nonspecific binding. Various dilutions of samples and standards were incubated in the plate overnight; then detecting antibody was added to each well. After 1 h, HRP-streptavidin (Zymed Inc., San Francisco, CA) was added to each well. Following a 30 min equilibration, the TMB substrate was added and the plate was incubated in the dark for 30 min. The colorimetric reaction was stopped by addition of 0.18 M sulfuric acid. Plates were washed three times with wash buffer (50 mM Tris with 0.2% Tween-20, pH 7.2) between each step. All the steps were conducted at ambient temperature. The absorbance within each well of the plate was measured on an ELISA plate reader at 450 nm. The background absorbance, measured at 550 nm, was subtracted from all readings. The limit of detection for the kit was 0.1 ng/ml. Because all samples were diluted a minimum of 1:5 with assay buffer, the limit of detection for samples was 0.5 ng/ml. Samples were assayed in duplicate and at a minimum of two dilutions. All assays included a pooled serum sample with a known IFN-γ concentration to serve as a laboratory standard. The intraassay variation was <10% based on this laboratory standard.

Statistical analysis

Data are expressed as mean ± SEM. The effect of dietary fat at various times during listeriosis was tested by two-way ANOVA. When significant diet differences occurred ($p < 0.05$), treatment mean differences were identified by Fisher's protected LSD test at each time point. All analyses were conducted on a Macintosh computer using Version 1.03 of StatView II software (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Effect of dietary fat on serum IFN-γ during murine listeriosis

IFN-γ was detected in the blood 2 days after mice were injected with 10^5 live *L. monocytogenes* (Fig. 1). By day 7, IFN-

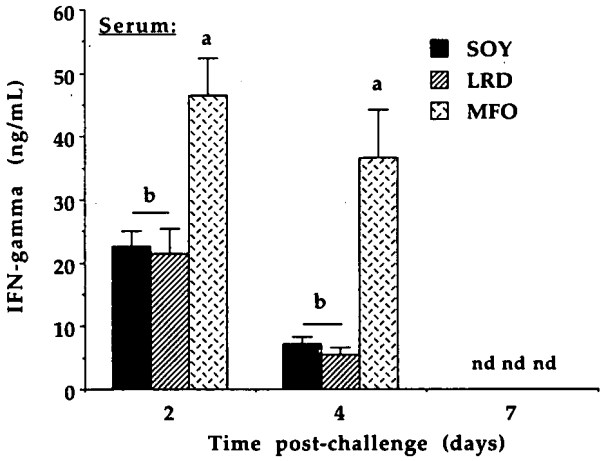

FIG. 1. The effect of dietary fat on the IFN-γ concentration in sera at various time points during murine listeriosis. Mice were infected with 10^5 live *Listeria monocytogenes*, and blood samples were collected 2, 4, and 7 days postinfection: The concentration of IFN-γ in sera samples was determined using a commercial ELISA kit. All samples were assayed in duplicate at two dilutions (1:5, 1:10). The limits of detection was 1 ng/ml of sample. Each result represents the mean ± SEM ($n = 8$ mice per treatment group). Means within each time point not sharing a letter are significantly different ($p < 0.05$). nd, not detectable; LRD, lard; MFO, menhaden fish oil; SOY, soybean oil.

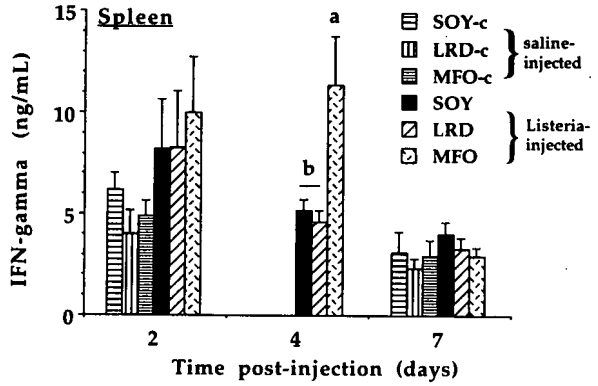

FIG. 2. The effect of dietary fat on the IFN-γ concentration in the spleen during murine listeriosis. Mice were challenged as described in Figure 1. Spleens were removed 2, 4, and 7 days postinfection, and then homogenized in sterile saline. IFN-γ concentration of these supernatants was determined by ELISA at two dilutions (1:2, 1:4). Each result represents the mean ± standard error for a group of 8 mice. Means within each time point not sharing a letter are significantly different ($p < 0.05$). LRD, lard; MFO, menhaden fish oil; SOY, soybean oil.

γ was no longer detectable in the blood. Mice fed the fish oil-containing diet (rich in n-3 PUFA) showed significantly higher ($p < 0.05$) amounts of IFN-γ in their blood on day 2 postchallenge compared with those fed the soybean oil-containing or lard-containing diet. On day 4 postchallenge, although the IFN-γ concentration in soybean oil-fed and lard-fed mice had declined compared with day 2, it remained high in those fed fish oil. The result was a 5-fold higher concentration of IFN-γ in the serum of mice fed fish oil compared with those fed soybean oil or lard ($p < 0.001$). No IFN-γ could be detected in the blood of mice injected with sterile saline either 2 or 4 days before sample collection (data not shown).

Effect of dietary fat on splenic IFN-γ during listeriosis

The concentration of IFN-γ in spleen homogenates during murine listeriosis is shown in Figure 2. In the spleen, IFN-γ was detected in both infected and noninfected mice (i.e., those injected with sterile saline). The concentration of IFN-γ in the spleen tended to increase 2 days after the bacterial challenge, but the variability of these data was high. By day 7, IFN-γ concentration in the spleen had declined such that there was no longer any indication of a *Listeria* challenge effect. There was no significant difference in the splenic IFN-γ concentration between diet treatments on day 2 and day 7 postchallenge. However, on day 4 postchallenge, a significantly higher ($p < 0.005$) level of IFN-γ was seen in mice fed fish oil compared with those fed soybean oil or lard.

Influence of dietary fat on the in vitro production and secretion of IFN-γ

In view of the higher levels of IFN-γ in the blood and spleen of fish oil-fed mice, we sought to determine if this was caused by an alternation in IFN-γ production. A second experiment was conducted following the same feeding and bacterial challenge protocol as in the first study. Splenocytes were isolated 4 days after a *Listeria* challenge. These immune cells were cultured alone or with mitogen (i.e., concanavalin A) to stimulate *in vitro* IFN-γ production and release. The secretion of IFN-γ during *in vitro* cell culture is shown in Table 2. *In vitro* IFN-γ secretion was not observed in the cells cultured without mitogen. Coculturing splenocytes with mitogen greatly increased the IFN-γ secretion. Splenocytes cultured with mitogen for 48 h had greater IFN-γ production than those cultured for 24 h. There were no significant differences in IFN-γ secretion by splenocytes from fish oil-fed and soybean oil-fed mice at either dose of mitogen tested (i.e., 1 and 10 μg/ml Con A) after either 24 or 48 h of culturing. Similar results were found when splenocytes were isolated from unchallenged mice (data not shown).

DISCUSSION

The early production of IFN-γ is believed to be critical in host defense against murine listeriosis.[36,37] Nakane et. al.[38] reported that endogenous IFN-γ appeared 16–24 h after *Listeria* infection in mice and then peaked at 48 h in the bloodstream and spleen. In our study, fish oil-fed mice showed significantly higher serum IFN-γ 2 days after infection compared with mice fed soybean oil or lard. The difference among dietary treatment groups was even greater on day 4 of the infection, when fish-oil fed mice had over 5-fold higher serum IFN-γ compared with mice fed the other diets. As far as we are aware, this is the first report of dietary fat influencing the *in vivo* IFN-γ response in

TABLE 2. EFFECT OF DIETARY FATS ON IN VITRO PRODUCTION OF IFN-γ BY MURINE SPLENOCYTES[a]

	No mitogen		*+1 mg/ml Con A*		*+10 mg/mL con A*	
Time	*24 h*	*48 h*	*24 h*	*48 h*	*24 h*	*48 h*
Dietary treatment			IFN-γ (ng/ml)[b]			
SOY	nd[c]	nd	27.1 ± 10.3	68.9 ± 15.6	42.8 ± 8.7	70.1 ± 8.4
MFO	nd	nd	27.1 ± 6.7	67.4 ± 12.3	34.0 ± 6.3	54.7 ± 8.8

[a]Spleens were harvested on day 4 postchallenge from mice infected with 10^5 CFU live *L. monocytogenes*. Single-cell suspensions of splenocytes (10^6/ml) were incubated at 37°C, 5% CO_2 with 0, 1, and 10 μg/ml Con A for 24 and 48 h.

[b]The concentrations of IFN-γ in cell supernatants were determined with a commercial ELISA kit. All samples were run in duplicate at two different dilutions. Data represent the mean ± SEM ($n = 5$ or 6). There were no significant differences between SOY and MFO treatment groups at either dose of mitogen and at either time point.

[c]nd, not detectable.

mice. The time course for the rise and fall of serum IFN-γ observed in our mice fed soybean oil or lard is consistent with that reported by others.[36,39] In contrast, the extended period for elevated serum IFN-γ observed for fish oil-fed mice is both an intriguing and a novel observation.

A significantly higher concentration of IFN-γ in crude spleen homogenates was also noted in fish oil-fed mice compared with mice fed soybean oil or lard. That this difference was only observed at day 4 is consistent with the time that we observed a log greater bacterial load in spleens of fish oil-fed mice compared with those fed soybean oil or lard.[35] It has been reported previously that at day 4 into a primary *Listeria* infection, the percentage of IFN-γ-producing cells in the spleen is at its greatest (i.e., ~20%).[40] Furthermore, these IFN-γ-producing cells were characterized as γ/δ T cells. In contrast, Poston and Kurlander[41] reported that splenic IFN-γ mRNA levels and serum IFN-γ in mice were both maximal on day 1 of a *Listeria* infection, decreasing steadily after that to barely detectable levels by days 4–6. We believe the primary reason for the difference in the cytokine response to *Listeria* infection has to do with the strain of mice used in these two studies. In the study of Hsieh et al.[40] and in our study, C3H mice, which are considered susceptible to *Listeria*, were used, whereas Poston and Kurlander used C57B1/6J mice, a resistant strain.[42] Qualitative and quantitative differences in the cytokine response between resistant versus susceptible strains of mice have been reported previously.[43]

Clearly, fish oil feeding appears to substantially alter the normal kinetics of the antilisterial response in the spleen and probably elsewhere. The response of fish oil-fed mice, rather than delayed, seems to be prolonged. This prolonged IFN-γ response may be a consequence of reduced PGE_2 production in the fish oil-fed mice. Recently, Hilkens et al.[44] proposed that the balance between IL-12 and PGE_2 production by accessory cells (i.e., macrophages) plays a critical role in the time-dependent control of IFN-γ production by $CD4^+$ T lymphocytes. Although we did not measure splenocyte PGE_2 production in these experiments, we[45] and others[46] have previously documented the ability of dietary fish oil to reduce murine splenocyte PGE_2 biosynthesis.

Although little is known of the nutritional modulation of IFN-γ production in mice, a few reports exist in which researchers explored the effect of certain fatty acids on IFN-γ production using either human subjects or human lymphocytes. For example, Purasiri et al.[47] conducted a study of patients with advanced colorectal cancer. They reported that the serum concentration of IFN-γ was significantly reduced, as were the levels of IL-1, IL-2, IL-6, and TNF-α, after 4 months of dietary supplementation with a combination of γ-linolenic acid, EPA, and DHA. The authors could not determine if this effect was specific for n-3 PUFA or any PUFA. In contrast, elevated IFN-γ production has been reported in postoperative cancer patients enterally fed n-3 PUFA for 16 days.[48] It is unclear if the effects observed were caused by n-3 PUFA or one of the other additives, as the feeding formula also contained nucleotides and arginine. Karsten et al.[49] demonstrated that palmitic acid, linoleic acid, and at certain concentrations, stearic acid and oleic acid could enhance *in vitro* release of IFN-γ by human peripheral lymphocytes. Among these free fatty acids, palmitic acid had the most potent effect and augmented release of IFN-γ twofold. In contrast to these studies, the results of our study clearly demonstrate that differences in dietary fat source alone influenced circulating IFN-γ during murine listeriosis. That the fish oil-fed mice responded differently from those mice fed soybean oil suggests that the effects observed are not associated with increased PUFA intake but specifically n-3 PUFA intake. It would be of interest to determine which of the fish oil-derived n-3 PUFA, EPA versus DHA, are most potent at modulating *in vivo* IFN-γ response in mice. Future studies with ethyl ester of EPA and DHA might shed some light on this issue.

A possible consequence of this dietary fish oil-induced increase in circulating IFN-γ could be enhanced MHC class II expression, since IFN-γ is a major upregulator of MHC class II expression. However, our laboratory[12] and others[22,50] have reported that n-3 PUFA reduces the expression of MHC class II molecules on immune cells. This immunomodulatory activity of n-3 PUFA may have physiologic relevance because of the critical role of antigen presentation in immune responses. Recently, we observed that dietary n-3 PUFA adversely affected the ability of mice to survive a lethal dose of *Listeria* infection.[35] We believe that the effect of n-3 PUFA on the immune response of mice to *Listeria* is achieved, at least in part, by the reduction in MHC class II expression on antigen-presenting cells, and we now believe that this is related to an alteration in the *in vivo* IFN-γ response.

The elevated concentration of IFN-γ in sera and spleens of fish oil-fed mice could be a consequence of enhanced production, reduced clearance, or both. To begin to address this issue, we measured the *ex vivo* secretion of IFN-γ by mitogen-stimulated splenocytes isolated from mice fed our experimental diets. We chose not to include the lard-fed mice in this experiment, since the *in vivo* responses of these mice were so similar to those of soybean oil-fed mice. Our study is novel in that the effects of dietary n-3 PUFA on the *in vivo* and *ex vivo* IFN-γ production were examined together. We deliberately chose to use splenocytes isolated from mice during the fourth day of listeriosis to maximize the probability that diet-induced differences could be detected *in vitro*. Furthermore, as stated previously, this is the optimal time during a primary *Listeria* response to find IFN-γ-producing cells.[40] Paradoxically, we failed to observe a significant difference in the *in vitro* production of IFN-γ by splenocytes isolated from mice fed fish oil compared with those fed soybean oil. Our findings sharply contrast with those of Vervliet et al.[51] They reported a 71%–100% increase in Con A-stimulated IFN-γ production when human peripheral blood leukocytes were pretreated with indomethacin, a PG synthesis inhibitor.

Thus, our data suggest that clearance of IFN-γ may be reduced or delayed by feeding mice fish oil. The binding of IFN-γ to its receptor and the subsequent endocytosis of the complex are believed to be the primary route of IFN-γ clearance *in vivo*.[52] Therefore, our observations suggest that dietary n-3 PUFA may be reducing the expression or function of the IFN-γ receptor. Consistent with this scenario is the reduced MHC class II molecule expression observed in mice on fish oil feeding. In other words, lower expression of MHC class II molecules in the presence of higher concentrations of IFN-γ during murine listeriosis is not paradoxical if we can demonstrate that fish oil feeding lowers the expression or function of IFN-γ receptors.

Information on the influence of dietary fat on cytokine receptors is limited to the IL-2 receptor. It has been reported that

the n-3 PUFA, DHA, and EPA decrease IL-2 receptor expression,[53] although others have failed to find this effect.[7,54] The effect of fatty acids on the IFN-γ receptors is unexplored, but some indirect data do exist. Somers et al.[8] have shown that macrophages from mice fed fish oil were hyporesponsive to IFN-γ during activation for tumoricidal activity. The same laboratory group reported that macrophages from mice fed menhaden fish oil had slightly decreased protein kinase C activity compared with macrophages from mice fed safflower oil.[55] The authors suggested that changes in receptor-mediated signal transduction pathways may explain the reduced responsiveness of macrophages from fish oil-fed mice to IFN-γ. Unfortunately, the possibility of a fish oil-induced reduction in IFN-γ receptor expression was not explored by these researchers. Interestingly, pretreatment of vascular smooth muscle cells with EPA or DHA has been shown to significantly reduce the binding of platelet-derived growth factor to its receptor.[56] This effect was not observed with linoleic or oleic acid pretreatment and did not alter the binding affinity (K_d) of the receptor. Further studies on the relationship of n-3 PUFA and the expression and function of IFN-γ receptors are planned, and data from these studies may improve our understanding of the mechanism(s) by which dietary n-3 PUFA alter immune responses.

In summary, the results of this study show that the dietary fat source influences the *in vivo* IFN-γ response in mice during listeriosis. Fish oil-fed mice have higher concentrations of IFN-γ in the serum and spleen compared with mice fed soybean oil or lard. Fish oil feeding appears to prolong as well as amplify the *in vivo* IFN-γ response. Paradoxically, we have documented lower expression of MHC class II molecules in fish oil-fed mice. *In vitro* production of IFN-γ by isolated splenocytes was similar among treatment groups. To resolve these discrepancies in our findings, we suggest that these diet-induced changes in circulating IFN-γ may be a consequence of alterations in the expression or function of IFN-γ receptors. Further studies need to be conducted to directly test this novel hypothesis.

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